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Differential response of ectomycorrhizal and saprotrophic fungal mycelium from coniferous forest soils to selected monoterpenes

Katherine E. Ludley [1,2], Clare H. Robinson [2], Sue Jickells [3], Paul M. Chamberlain [1] and Jeanette Whitaker [1]

[1] Centre for Ecology and Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster, LA1 4AP, UK*

[2] School of Earth, Atmospheric and Environmental Sciences, University of Manchester, Williamson Building, Oxford Road, Manchester, M13 9PL, UK

[3] Department of Forensic Science and Drug Monitoring, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NH, UK

Author for correspondence: Jeanette Whitaker

Tel: +44 1524 595800

Fax: +44 1524 61536

Email: jhart@ceh.ac.uk

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Abstract

The mycelia of saprotrophic (SP) and ectomycorrhizal (ECM) fungi occur throughout the upper soil horizons in coniferous forests and could therefore be exposed to high concentrations of monoterpenes occurring in the needle litter of some tree species. Monoterpenes are mycotoxic and could potentially affect fungi that are exposed to them in the litter layers. In order to investigate whether monoterpenes typical of coniferous litters could influence fungal communities, we analysed the monoterpene content of freshly fallen needles of *Pinus sylvestris*, *Picea abies* and *Picea sitchensis.* The most abundant monoterpenes were found to be α-pinene, β-pinene and 3-carene. We evaluated the effects of these three monoterpene vapours on the biomass production of 23 SP isolates and 16 ECM isolates. Overall, 75 % of ECM isolates and 26 % of SP isolates were significantly inhibited by at least one of the monoterpene treatments and both intra- and interspecific variations in response were observed. Monoterpene concentrations are highest in surface litters. The differential effects on fungal taxa may influence the spatial and temporal distribution of fungal community composition, indirectly affecting decomposition and nutrient cycling, the fundamental ecosystem processes in which fungi have a key role in coniferous forest soils.

Keywords: monoterpenes, ectomycorrhizal fungi, saprotrophic fungi, community structure, litter layer

1. Introduction

Coniferous trees dominate the boreal zone as well as significantly contributing to areas of temperate forest. In coniferous trees, monoterpenes, along with

sesquiterpenes and diterpenes, form the oleoresin that acts as the trees' defence system, protecting the tree against herbivory, insect attack and fungal infection. The needles of most coniferous tree species contain high concentrations of monoterpenes. These concentrations can remain relatively high when needles fall from the branches and enter the litter layer (Wood et al., 1995; Kainulainen and Holopainen, 2002; Kainulainen et al., 2003) and their volatility and generally insolubility in water are likely to result in their accumulation in the vapour phase in surface litters (Wilt et al., 1993a). Monoterpenes have widely reported antibacterial and antifungal properties (Payne et al., 2001; Guynot et al., 2003; Hammer et al., 2003; Suhr and Nielsen, 2003), and their mode of action appears to be through disruption of plasma membranes (Andrews et al., 1980; Trombetta et al., 2005). Monoterpene vapours accumulating in litter layers have the potential to equilibrate with lipid membranes and, in this way, influence the growth and activity of organisms living in coniferous needle litters.

In coniferous forests, which tend to be on nutrient-poor and acidic soils, it is the fungal community that plays the major role in decomposition and nutrient and carbon cycling. The mycelia of both litter-degrading saprotrophic (SP) fungi and ectomycorrhizal (ECM) fungi occur in the surface litter layer (the L horizon) and the underlying F (fermentation) horizon (O'Brien et al., 2005; Genney et al., 2006; Lindahl et al., 2007). Some sensitivity to monoterpenes by ECM has been observed in previous studies. Melin and Krupa (1971) suggested that monoterpenes occurring in the roots of coniferous trees are involved in maintaining the symbiotic relationship with their associated fungi. They demonstrated the inhibition of growth of two ECM species by a range of monoterpenes and proposed that this inhibitive effect enabled

76 the host tree to control the growth of the fungus. Koide et al. (1998) also demonstrated
77 that pure monoterpene vapours, and dead needles of *Pinus resinosa*, generally had an
78 inhibitory effect on ECM growth.
79
80 While the effects of monoterpenes have been tested on a small number of ECM
81 isolates in these two earlier studies, so far no studies have been carried out on litter-
82 decomposing SP fungi, which are also present in the upper soil horizons. Differential
83 effects on fungal taxa or functional groups could indirectly affect productivity,
84 decomposition rate, and nutrient cycling by influencing fungal community structure.
85 Monoterpenes are lost from the litter as the litter ages (Wilt et al., 1993b; Kainulainen
86 et al., 2003) and therefore will be at their highest concentrations in surface litters.
87 Differential effects of monoterpenes on fungal taxa could therefore also influence the
88 temporal and spatial structure of the fungal community by influencing their
89 distribution through the litter and soil horizons.
90
91 The aim of this study was to test the effects of specific monoterpenes on a wide range
92 of isolates of both SP and ECM fungi typical of coniferous forest ecosystems, and to
93 determine whether there were differences in response between the two functional
94 groups of fungi studied, between individual species, or between different isolates of a
95 single species (*Mycena galopus*). The monoterpenes in fresh litters of *Pinus sylvestris*,
96 *Picea abies* and *Picea sitchensis* were identified and the most abundant monoterpenes
97 selected for testing. The effects of the vapours of single, pure monoterpenes on the
98 biomass production of a range of fungal cultures grown in liquid nutrient medium
99 were evaluated.
100

2. Materials and Methods

2.1. *Monoterpene analysis*

Freshly fallen needles were collected on plastic sheets placed on the forest floor over a period of approximately eight weeks from monoculture stands of *Pinus sylvestris* (L.), *Picea abies* (L.) Karst, and *Picea sitchensis* (Bong.) Carr. at Grizedale Forest, Cumbria, UK (National Grid Reference SD 345944). For each species, collected needles were pooled and eight samples (approximately 4 g fresh weight) were taken for analysis. Each sample was air-dried for 24 hours at room temperature and then homogenised in an electric coffee mill (Braun Aromatic KSM2) for up to 20 seconds, until there were no whole needles remaining in the sample. Approximately 0.6 g of homogenised needles from each sample were used for the monoterpene extraction and the remainder of the sample was dried at 105°C for 24 hours to obtain the dry weight conversion factor. The fraction of needles used for monoterpene extraction was weighed into a clear glass vial and 50 µl of internal standard of 0.5 mg ml^{-1} adamantane (Sigma-Aldrich, Gillingham, Dorset, UK) in hexane was vortex mixed with the needles. The standard was left to equilibrate for 30 min before adding 4 ml of hexane, vortex mixing, and placing on a Belly Dancer shaker (Stovall Life Science Inc., Greensboro., North Carolina, USA) speed setting 5, for 90 min in darkness. The extract was then filtered through a 1 cm plug of cotton wool to remove particulate material, and analysed by gas chromatography with flame ionisation detection (GC). GC analysis was carried out on an Agilent Technologies 6890N gas chromatograph (South Queensferry, West Lothian, UK). Samples of 1 µl were injected onto a CP SIL 5 column with on-column injection (Varian Inc., Walton-on-Thames, UK), column

126 length 60 m, internal diameter 0.32 mm, film thickness 25 μm. The detector
127 temperature was 350°C, and the carrier gas used was H_2 with a flow rate of 40 cm s^{-1}.
128 The oven temperature programme was 50°C for 2 min, ramping at 5°C min^{-1} to
129 115°C and then at 25°C min^{-1} to 325°C and held for 15 min.
130
131 Quantification was achieved by referring to the internal standard, assuming a 1:1
132 response for internal standard and extracted monoterpenes. Identification of individual
133 compounds was carried out by comparing retention times with monoterpene standards
134 (Sigma-Aldrich, Gillingham, Dorset, UK), and also using gas chromatography-mass
135 spectrometry (GC-MS) analysis. GC-MS analysis was carried out on an Agilent
136 Technologies 6890 GC connected to an Agilent 5973N Mass Selective Detector.
137 Samples of 1 μl were injected via an on-column inlet onto a DB-1 MS column (J & W
138 Scientific, USA), length 30 m, internal diameter 0.32 mm, film thickness 0.25 μm.
139 The injector port temperature was 50°C with an oven temperature programme of 50°C
140 for 3 min, ramping at 5 °C min^{-1} to 100°C and then ramping at 25°C min^{-1} to 300°C.
141 Solvent delay was set at 4 min. Electron impact spectra were acquired at 70 eV and
142 the MS was run in SCAN mode (50- 550 amu at 2.94 scans per second). Data
143 processing was carried out using HP Chemstation.
144
145 2.2. *Fungal cultures*
146
147 The majority of the ECM and SP cultures used were obtained from the collection of
148 Juliet C. Frankland, which is maintained at the Centre for Ecology and Hydrology,
149 Lancaster. Isolates were also obtained from a collection at the University of Cardiff.
150 New cultures were isolated from basidiome tissue collected in autumn 2003 from

151 Grizedale Forest, Cumbria, UK, and Gisburn Forest, Lancashire, UK (National Grid
152 Reference SD 750588) from plots of *P. sylvestris*, *P. sitchensis* and *P. abies*. Details
153 of individual cultures are given in Table 1.
154
155 SP cultures were maintained on Potato Dextrose Agar medium (PDA) (Oxoid,
156 Hampshire, UK) and ECM cultures were maintained on modified Melin-Norkrans
157 agar medium (MMN). MMN was made up with 1 l of distilled water containing (g):
158 $(NH_4)HPO_4$, 0.25; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.15; D-glucose, 10.0; malt extract,
159 3.0, Agar Technical Number 3 (Oxoid, Hampshire, UK) 10.15; and (mg) $CaCl_2.6H_2O$,
160 50; NaCl, 25; thiamine, 0.1, FeEDTA, 16.3; citric acid, 16.3; adjusted to pH 5.5 and
161 autoclaved at 121°C for 15 minutes. Cultures were stored in darkness at 20 ± 2°C.
162
163 2.3. *Preparation of fungal inoculum*
164
165 Prior to exposure experiments, fungal inocula were prepared by taking three plugs (5
166 mm diameter) from cultures growing on either PDA (SP cultures) or MMN (ECM
167 cultures) and placing them in 75 ml of either 3% w/v malt extract liquid medium
168 (MEL) (Sigma-Aldrich, Gillingham, Dorset, UK) or liquid MMN respectively, in
169 250 ml Erlenmeyer flasks. Liquid MMN contained per litre of distilled water (g):
170 $(NH_4)HPO_4$, 0.5 ; KH_2PO_4, 0.3; $MgSO_4.7H_2O$, 0.14; $CaCl_2.6H_2O$, 0.05; D-glucose,
171 10.0; and (mg) NaCl, 25; $ZnSO_4$, 3.0; thiamine, 0.1; FeEDTA, 37.5; citric acid, 37.5;
172 adjusted to pH 5.5.
173
174 After four weeks at 20 ± 2°C in darkness, the mycelia from two flasks of each isolate
175 were combined and placed in the sterile cup of a liquidiser with 40 ml of sterile

176 distilled water and homogenised. Aliquots (2 ml) of homogenate were used to
177 inoculate 23 ml of either MEL or liquid MMN in 100 ml Katz flasks. To the central
178 well of each flask was then added 300 µl of either: filter-sterilised liquid α-pinene, β-
179 pinene or 3-carene (purity > 95%, Sigma-Aldrich, Gillingham, Dorset, UK). In
180 control flasks the central well was left empty. The mean mass of added inoculum was
181 determined by measuring the dry weight of three replicates of inoculum (2 ml) for
182 each isolate. Three replicates of each isolate were prepared for each treatment and
183 flasks were sealed with silicon rubber Suba seals (William Freeman Ltd, Barnsley,
184 South Yorkshire, UK). As the monoterpenes are volatile, the cultures would then be
185 exposed to monoterpene vapours in the headspace of the sealed flask.
186
187 During the exposure, cultures were incubated at 15°C in darkness and aerated once a
188 week by placing the cultures in a sterile cabinet and replacing the Suba seal with a
189 sterile cotton wool bung for 45 min. After two weeks, the liquid monoterpene
190 remaining in the well was removed and replaced with a further 300 µl. Mycelia were
191 harvested after six weeks (SP) and eight weeks (ECM) and dry weight biomass was
192 measured by drying at 105°C for 24 hours. The growth form of each isolate was
193 recorded before harvesting.
194
195 Replicate exposures were carried out, comparing either ECM or SP isolates. One of
196 the experiments compared nine different isolates of the SP fungus *M. galopus*,
197 including three isolates of *M. galopus* var. *galopus*, three isolates of *M. galopus* var.
198 *candida* and three isolates of *M. galopus* var. *nigra*. In total, 16 ECM isolates
199 (including 13 different species), and 23 SP isolates (14 species) were tested.
200

2.4. *Measurement of headspace monoterpene concentrations*

In order to estimate the monoterpene concentration in the headspace of the Katz flasks, headspace samples of 3 ml were taken through the Suba seal lid from Katz flasks set up without fungal inoculum (to avoid contamination of the experimental series of flasks). Samples were taken every two days after monoterpene addition for ten days and injected directly onto a Perkin Elmer 8500 gas-chromatograph (GC). The GC was fitted with a 1.5 ml sample loop with an unheated injector port and oven temperature programme starting at 75°C, held for 1 min and ramped at 5°C min^{-1} to 90°C. The column was a 1.8m x 2mm ID packed column (10% AT - 1000 on Chromosorb W-AW, 80/100) (Alltech, Carnforth, Lancashire, UK) and data were analysed using Clarity software (DataApex, Prague, Czech Republic). Mixed standard gases were of 10 ppm and 60.1 ppm α-pinene and β-pinene in nitrogen (CK Gas Products Ltd, Hook, Hampshire, UK).

2.5. *Data analysis*

Data were analysed using Statistica (V6, StatSoft Inc, Tulsa, OK, USA). The raw biomass data were tested for a normal distribution using the Kolmogorov-Smirnov test for normality and found to be normally distributed. The effects of the three monoterpenes were then analysed for each species separately using analysis of variance (ANOVA), and means were compared between treatments using a post-hoc Tukey-HSD test. In order to compare the overall response of SP with ECM isolates, data were log transformed before ANOVA.

3. Results

3.1. *Monoterpene extractions*

In *P. sylvestris* the dominant monoterpenes were found to be α-pinene and 3-carene, which contributed 46% and 37% respectively to the total monoterpene content of freshly fallen litter. In *P. abies*, α-pinene and β-pinene were clearly dominant, contributing 19% and 56% to the total monoterpene content. The most abundant monoterpene in *P. sitchensis* litter was limonene, but the total monoterpene concentration was several times lower than the concentrations found in litters of the other two species (100 ± 5 μg g^{-1} dry wt compared with 1531 ± 96 and 1175 ± 122 μg g^{-1} dry wt in *P. abies* and *P. sylvestris* respectively, data not shown). Consequently, α-pinene, β-pinene and 3-carene were chosen for use in exposure experiments.

3.2. *Exposure of fungal isolates to monoterpenes*

Monoterpene concentrations in the headspace of the flasks remained fairly constant, at around 30 mg l^{-1}. In order to compare the relative effects of the monoterpene treatments on different isolates, Figs 1 to 3 present the mean final biomass of each isolate exposed to the monoterpene treatments as a percentage of mean final control biomass. Statistical analysis was carried out on raw biomass data, rather than percentages, and statistically significant results are shown in Figs 1 to 3. In order to compare growth between the different isolates, final mean control biomass is also presented in Tables 2 to 4.

3.3. *Exposure of ECM isolates*

Twelve of the 16 ECM isolates tested were significantly inhibited by the presence of monoterpene vapours (Fig. 1). *Amanita muscaria*, *Amanita rubescens*, *Cenococcum geophilum*, *Laccaria laccata*, *Paxillus involutus*, *Suillus grevillei*, and *Thelephora terrestris* were significantly inhibited by all three monoterpene treatments. Biomass production of *Hebeloma crustuliniforme* was significantly inhibited by the 3-carene and β-pinene treatments ($P<0.05$), and *Boletus badius* was significantly inhibited in the presence of β-pinene ($P<0.05$).

The most severely inhibited species were *P. involutus* and *A. muscaria* ($P < 0.001$ for all treatments). Two different isolates were tested for each of these species. Both *P. involutus* isolates had a final mean biomass of less than 20% of control cultures under all treatments. However, biomass of *A. muscaria* isolate 1 was reduced to between 11% and 17% of control biomass, while the biomass of isolate 2 varied between 34% and 51% of control biomass under the monoterpene treatments.

In addition to an inhibition of growth, differences in the growth form of some of the sensitive isolates were observed. In control cultures of *A. muscaria*, *A. rubescens*, *P. involutus* and *S. grevillei*, the mycelium formed a dense mat floating on the surface of the medium, whereas when these isolates were exposed to monoterpenes, little or no mycelium grew above the surface.

In control cultures of *C. geophilum* (isolate 2 in particular), *L. laccata* and *T. terrestris*, while there was some mycelium visible growing above the surface of the

liquid nutrient medium, a greater proportion of mycelium grew submerged. These three isolates did show significant inhibition under all monoterpene treatments and produced a mean final biomass of between 50% and 64% (*C. geophilum* isolate 2), 28% and 54% (*L. laccata*) and 55% and 66% (*T. terrestris*) of control biomass under the different monoterpene treatments (Fig. 1).

Data for *Lactarius rufus* were not conclusive because of a high degree of variability in the growth of the control cultures (Table 2). This variability may have been caused by sensitivity to the homogenisation procedure as, in fact, only one of the controls for *L. rufus* seemed to produce any new mycelium after inoculation.

Tricholoma aurantium and *Amanita vaginata* were not significantly affected by any of the monoterpene treatments (Fig. 1, $P>0.05$). There were no significant differences between individual monoterpene treatments for any isolates.

3.4. *Exposure of SP isolates*

The SP species generally grew faster than the ECM and were harvested after six rather than eight weeks. Only five of the 17 SP isolates tested showed significant inhibition by any of the monoterpene treatments (Fig. 2). As was found for the exposures of ECM isolates, there were no significant differences in the response of SP isolates between individual monoterpene treatments.

Three SP isolates were significantly inhibited by all three monoterpene treatments (Fig. 2); both isolates of *Mycena galopus* ($P<0.05$ for all treatments) and *Mycena*

301 *galopus* var. *candida* ($P<0.01$ for all treatments). Several of the isolates, such as
302 *Marasmius androsaceus* and *Cystoderma amianthinum* appeared to show reduced
303 growth in the presence of monoterpenes (Fig. 2) but the reduction was not significant
304 ($P>0.05$). *Mycena cinerella* also appeared to show reduced growth under all three
305 monoterpene treatments but this was only significant for α-pinene ($P<0.05$). The
306 variable growth rates of the control cultures of these isolates, grown without
307 monoterpene vapours (Table 3), may explain why such apparent reductions were not
308 statistically significant. *Marasmius epiphyllus* also showed reduced growth under all
309 monoterpene treatments, although inhibition was only significant under one of the
310 monoterpene treatments (β-pinene, $P<0.05$), where biomass was approximately 30%
311 of control (Fig. 2). For this isolate, there was more variability in growth rate in the
312 monoterpene-treated cultures than in control cultures, which may have contributed to
313 the lack of statistical significance of the apparent reduction in biomass under the other
314 monoterpene treatments.
315
316 A further experiment was set up to compare nine different isolates of *M. galopus*.
317 Three of the isolates used had already been tested (*M. galopus* isolate 2, *M. galopus*
318 var. *candida* isolate 1 and *M. galopus* var. *nigra* isolate 1) and were tested again to see
319 whether results were reproducible. *M. galopus* isolate 2 had been tested twice before,
320 once showing significant inhibition under all treatments and once showing no
321 significant inhibition. When it was tested for the third time, with the other *M. galopus*
322 isolates, it was not significantly inhibited by any of the monoterpene treatments (Fig.
323 3), and neither were the two new isolates of *M. galopus* (isolates 3 and 4). There was
324 a difference in growth between the *M. galopus* controls, with *M. galopus* isolate 4
325 appearing to be slower growing than the other two isolates (Table 4).

Of the three isolates of *M. galopus* var. *candida*, only one isolate showed a significant response to the presence of monoterpenes; the previously tested *M. galopus* var. *candida* isolate 1 again showed a significant reduction in biomass in the presence of α-pinene (Fig. 3a, $P<0.05$). Isolate 2 of *M. galopus* var. *nigra* showed a significant inhibition under all three treatments (Fig. 3, $P<0.01$). The previously tested *M. galopus* var. *nigra* isolate 1 again showed no significant inhibition.

The growth form of the SP isolates was also observed before harvesting. While some mycelium was visible growing above the surface of the liquid medium for most SP isolates, the bulk of the fungal material was always submerged.

3.5. *Comparison of response of ECM with SP isolates*

The overall response of ECM isolates was compared with that of SP isolates using ANOVA (performed on log-transformed data). A significant interaction between treatment and functional group was detected ($P<0.05$), with monoterpenes having a less inhibitory effect on SP isolates overall than on ECM isolates.

4. Discussion

4.1. *Monoterpene extractions*

Studies of monoterpene contents of both green and senescent needles of various coniferous tree species have been carried out by other authors. The most abundant

monoterpenes in green needles of *P. sylvestris* are consistently α-pinene and 3-carene (Sjödin et al., 2000; Kainulainen and Holopainen, 2002). Slightly different proportions to those found here were reported in needles of *P. sylvestris* from Finland, with α-pinene contributing 51% and 3-carene 17% of the total monoterpene content (Kainulainen and Holopainen, 2002), compared with 46% and 37% found here. This difference may be due to geographical variation which has been shown to occur (Janson, 1993).

In a review collating data on monoterpene emissions from different conifers (Geron et al., 2000), α-pinene was found to contribute 45% of total emissions from *P. abies* and β-pinene 12%. This is similar to the 56% and 19% reported here.

In this study, monoterpene concentrations in needles of *P. sitchensis* were lower than in the other two species. Data from the literature indicate comparable monoterpene emission concentrations between *P. sitchensis* and *P. abies* (Hayward et al. 2004; Grabmer et al., 2006), however, needle monoterpene concentrations in *P. sitchensis* have generally been expressed as percentages of total monoterpenes rather than actual concentrations (e.g. Hrutifiord et al., 1974), thus we have been unable to compare our findings with those of other studies.

4.2. *Exposure of fungal isolates to monoterpenes*

The response of both ECM and SP isolates to the presence of monoterpenes common in the natural environment was varied, and there was a significant difference in response between the two functional groups, with SP isolates overall being

significantly less sensitive than ECM. There was no significant difference between the effects of the different monoterpenes.

Of the ECM isolates tested in this study, 75% were shown to be sensitive to monoterpene vapours when grown in pure liquid culture. In a study by Koide et al. (1998), all eight species of ECM that were tested were significantly inhibited by a mixture of α-pinene and β-pinene, suggesting that ECM fungi are generally sensitive to common monoterpenes.

Changes in the growth pattern of fungal colonies grown on solid nutrient media and exposed to α-pinene and β-pinene were observed by Koide et al. (1998). With two ECM species growing on Hagem agar, Melin and Krupa (1971) also observed, "compact restricted colonies with very little or no loose mycelia in the outer section," in fungi exposed to a range of monoterpenes and sesquiterpenes. Corresponding changes were observed in the current study.

Some of the most severely inhibited ECM species were *A. muscaria*, *A. rubescens*, *P. involutus* and *S. grevillei*. Control cultures of these species produced a significant proportion of their mycelium floating on the surface of the liquid medium. The low aqueous solubility of monoterpenes may protect mycelium growing below the surface of the liquid medium and this may explain why some of the surface growers such as *P. involutus* and *A. muscaria* were more sensitive. Control cultures of *A. vaginata* also grew mainly floating on the surface, and although there was a reduction in the proportion of mycelium visible above the surface in monoterpene exposed cultures, there was no significant reduction in overall biomass production.

401 The isolates in this study were exposed to high monoterpene concentrations of up to
402 30 mg l^{-1} in the headspace of the flasks. Actual environmental concentrations of
403 monoterpenes in the air-space of needle litters are difficult to quantify precisely. For
404 example, Paavolainen et al. (1998) measured monoterpenes in soil air by placing
405 passive diffusive samplers within the litter, but these gave quantities of monoterpenes
406 collected over an eight week period which are difficult to interpret in terms of actual
407 exposure levels. Total monoterpene concentrations in the soil (mineral and litter layer)
408 atmosphere under *P. sylvestris* were measured at 106 mg m^{-3} (0.1 mg l^{-1}) (Smolander
409 et al., 2006), but concentrations building up in the litter layer itself will be much
410 higher. For example, Wilt et al. (1993a) measured total monoterpene concentrations
411 of 3.56 mg l^{-1} in the air space of a sealed glass vessel filled with *Pinus monophylla*
412 litter. The experimental concentrations to which the fungal isolates were exposed in
413 the Katz flasks were therefore around ten times the concentration that has been
414 measured in the air-space of coniferous litter.
415
416 The highest concentrations of monoterpenes occur in surface litters, where
417 monoterpenes are constantly volatilising from freshly fallen needles. As the litter ages
418 and becomes incorporated into lower horizons, monoterpenes will virtually disappear
419 (Kainulainen and Holopainen, 2002). A number of studies have examined the vertical
420 distribution of fungi through the litter and soil horizons, mostly with regard to ECM
421 species, and all have demonstrated that certain species will segregate into particular
422 horizons, associated with particular categories of organic matter (Dickie et al., 2002;
423 Rosling et al., 2003; Tedersoo et al., 2003; Genney et al., 2006; Lindahl et al., 2007).
424 Lindahl et al. (2007) found that there was a distinct change in fungal community
425 composition between the surface litter, or L horizon, consisting of intact needles, and

426 the lower, more decomposed F horizon. They found that the litter-decomposing SP
427 fungi *Mycena* and *Marasmius* occupied the L horizon, whereas the mycelium of ECM
428 species tended to occur in the F horizon. O'Brien et al. (2005) similarly detected
429 sequences of *Mycena* species in the L horizon, while species of *Russula* were more
430 frequent in the deeper organic horizons and mineral soil.
431
432 There are many factors that will influence the depth at which different fungal taxa
433 tend to occur. These may be associated with substrate quality or with environmental
434 conditions that vary according to depth, such as temperature or moisture content
435 (Osono et al., 2006). The variation in sensitivity to monoterpenes shown by the taxa
436 tested here suggests that there is nevertheless the potential for monoterpenes in the
437 litter layer to be a contributory factor. Our finding, that the litter-decomposing isolates
438 tested were less sensitive to monoterpenes than ECM isolates, would correspond with
439 the pattern of distribution observed by Lindahl et al. (2007) and O'Brien et al. (2005),
440 with SP fungi occurring in the layers containing the younger litter material and the
441 highest monoterpene concentrations.
442
443 The intraspecific variation in response shown by *M. galopus* adds further complexity
444 to the possible influence of monoterpenes on fungal community structure. Cairney
445 (1999) reviewed studies where multiple isolates of the same ECM taxa were used to
446 test various physiological characteristics. While few studies used more than five
447 isolates of any species, it is clear that a large amount of intraspecific physiological
448 variation exists. This variation could be the result of adaptation to conditions in
449 different geographical areas, or to more localised heterogeneities (Cairney, 1999). It is
450 not possible from this study to assign the cause of the observed intra-specific variation

to adaptation to any specific environmental factors. It is nonetheless important to acknowledge that such variability can and does exist and should be taken into account in any further studies.

These exposure experiments were designed to test whether there is likely to be significant variation in the response of fungi to monoterpenes. It is recognised that the use of liquid culture represents an artificial system, meaning that mycelial growth rates and forms and the nature of rhizodeposition are unlikely to reflect those that occur in natural substrata. In addition, the actual growth forms of the isolates in liquid nutrient medium may have affected their exposure levels, as isolates growing on the surface will have been exposed to higher concentrations of monoterpenes in the headspace of the container. Despite these drawbacks, the results indicate that there is considerable variation between the species in their sensitivity to monoterpenes, and further investigation of the effects of monoterpenes on fungi in more realistic conditions would be justified.

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Figure 1. Growth of ectomycorrhizal fungal isolates exposed to vapours of (a) α-pinene, (b) 3-carene and (c) β-pinene. Data are expressed as final biomass dry wt as % of control biomass dry wt (mean ± SEM, n=3). Significant difference from control, calculated on raw biomass data, is indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001), Tukey's HSD. 1a, *Amanita muscaria* isolate 1; 1b, *Amanita muscaria* isolate 2; 2, *Amanita rubescens*; 3, *Amanita vaginata*; 4, *Boletus badius*; 5a, *Cenococcum geophilum* isolate 1; 5b, *Cenococcum geophilum* isolate 2; 6, *Hebeloma crustuliniforme*; 7, *Laccaria amethystina*; 8, *Laccaria laccata*; 9, *Lactarius rufus*; 10a, *Paxillus involutus* isolate 1; 10b, *Paxillus involutus* isolate 2; 11, *Suillus grevillei*; 12, *Thelephora terrestris*; 13, *Tricholoma aurantium.*

Figure 2. Growth of saprotrophic fungal isolates exposed to vapours of (a) α-pinene, (b) 3-carene and (c) β-pinene. Data are expressed as final biomass dry wt as % of control biomass dry wt (mean ± SEM, n=3). Significant difference from control, calculated on raw biomass data, is indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001), Tukey's HSD. 1, *Collybia butyracea*; 2, *Collybia maculata*; 3, *Collybia peronata*; 4, *Cystoderma amianthinum*; 5, *Marasmius androsaceus*; 6, *Marasmius epiphyllus*; 7, *Mycena cinerella*; 8a, *Mycena galopus* isolate 1; 8b, *Mycena galopus* isolate 2; 9, *Mycena galopus* var. *candida*; 10 *Mycena galopus* var. *nigra*; 11, *Mycena purpureofusca*; 12, *Mycena vulgaris*; 13, *Mycena epipterygia*; 14, *Mycena rubromarginata*; 15, *Mycena sanguinolenta*; 16, *Rickenella fibula*.

Figure 3. Growth of different isolates of *Mycena galopus* exposed to vapours of (a) α-pinene, (b) 3-carene and (c) β-pinene. Data are expressed as final biomass dry wt

as % of control biomass dry wt (mean ± SEM, n=3). Significant difference from control, calculated on raw biomass data, is indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001), Tukey's HSD. 1, *Mycena galopus* isolate 2; 2, *Mycena galopus* isolate 3; 3 , *Mycena galopus* isolate 4; 4, *Mycena galopus* var. *candida* isolate 1; 5, *Mycena galopus* var. *candida* isolate 2; 6, *Mycena galopus* var. *candida* isolate 3; 7, *Mycena galopus* var. *nigra* isolate 1; 8, *Mycena galopus* var. *nigra* isolate 2; 9, *Mycena galopus* var. *nigra* isolate 3.

Table 1: List of fungal species and isolates tested. Culture code numbers refer to the number assigned to them in the collection of Dr Juliet C. Frankland unless otherwise stated. Where there is no culture code number, cultures were from freshly collected basidiome tissue.

Species	Culture code numbers	Substrate or tree species (where recorded)	Date isolated (where recorded)
Ectomycorrhizal isolates			
Amanita muscaria (L.) Lam.			
A. muscaria (Isolate 1)	Cardiff (A mus 0044)	Unknown	1985
A. muscaria (Isolate 2)	M9120	Unknown	1979
Amanita rubescens Pers.	Cardiff (Ar 006)	No details	1988
Amanita vaginata (Bull.) Lam.		*P. sylvestris*	2003
Boletus badius (Fr.) Fr.	M10627	Unknown	
Cenococcum geophilum Fr.			
C. geophilum (Isolate 1)	Cardiff (C geo 014)	Spruce litter	
C. geophilum (Isolate 2)	M10630	Unknown	1983
Hebeloma crustuliniforme (Bull.) Quél.	M10640	Unknown	1989
Laccaria amethystina Cooke	M8002	Unknown	1969
Laccaria laccata (Scop.) Cooke		Coniferous forest	2003
Lactarius rufus (Scop.) Fr.		*P. sitchensis*	2003
Paxillus involutus (Batsch) Fr.			
P. involutus (Isolate 1)		*P. sylvestris*	2003
P. involutus (Isolate 2)		Coniferous forest	2003
Suillus grevillei (Klotzsch) Singer	Cardiff (Se 010)	Unknown	
Thelephora terrestris Ehrh.	Cardiff (Thel t 12)	Unknown	1983
Tricholoma aurantium (Schaeff.) Ricken	M10661	*P. sitchensis*	1983
Saprotrophic isolates			
Collybia butyracea (Bull.) P. Kumm.	M9269 (b)	Mixed woodland litter	1981
Collybia maculata (Alb. & Schwein.) P. Kumm.	M10277	*P. sitchensis* litter	1989
Collybia peronata (Bolton) P. Kumm.	M10330	Unknown	1991
Cystoderma amianthinum (Scop.) Fayod	M9195	*P. sitchensis* litter	1977
Marasmius androsaceus (L.) Fr.	M10556	*P. sitchensis* litter	1998
Marasmius epiphyllus (Pers.) Fr.	M10263	Mixed woodland litter	1989
Mycena cinerella (P. Karst) P. Karst	M9235	Unknown	1980
Mycena epipterygia (Scop.) Gray	M9083	*P. abies* litter	1976
Mycena galopus (Pers.) P. Kumm			
M. galopus (Isolate 1)	M10500	*P. sitchensis* litter	1997
M. galopus (Isolate 2)	M10501	*P. sitchensis* litter	1997
M. galopus (Isolate 3)	M10502	*P. sitchensis* litter	1997
M. galopus (Isolate 4)	M10550	*P. sitchensis* litter	1998

M. galopus var. *candida* J. E. Lange			
M. galopus var. *candida* (Isolate 1)	M10505	*P. sitchensis* litter	1997
M. galopus var. *candida* (Isolate 2)	M9077	*P. abies* litter	1976
M. galopus var. *candida* (Isolate 3)	M10514	*P. sitchensis* litter	1997
M. galopus var. *nigra* Rea			
M. galopus var. *nigra* (Isolate 1)	M10504	*P. sitchensis* litter	1997
M. galopus var. *nigra* (Isolate 2)	M9092	*P. abies* litter	1976
M. galopus var. *nigra* (Isolate 3)	M10513	*P. sitchensis* litter	1997
Mycena purpureofusca (Peck) Sacc.	M9262b	*P. sylvestris* litter	1981
Mycena rubromarginata (Fr.) P. Kumm	M9286	*P. sylvestris* litter	1981
Mycena sanguinolenta (Alb. & Schwein) P. Kumm.	M10273	*P. sitchensis* litter	1989
Mycena vulgaris (Pers.) P. Kumm.	M10280	*P. sitchensis* litter	1989
Rickenella fibula (Bull.) Raithelh.	M10283	*P. sitchensis* litter	1989

Table 2. Growth (mg dry wt) of ectomycorrhizal isolates from control treatments after 8 weeks in liquid culture medium. Data represent the mean of three replicates (± SEM).

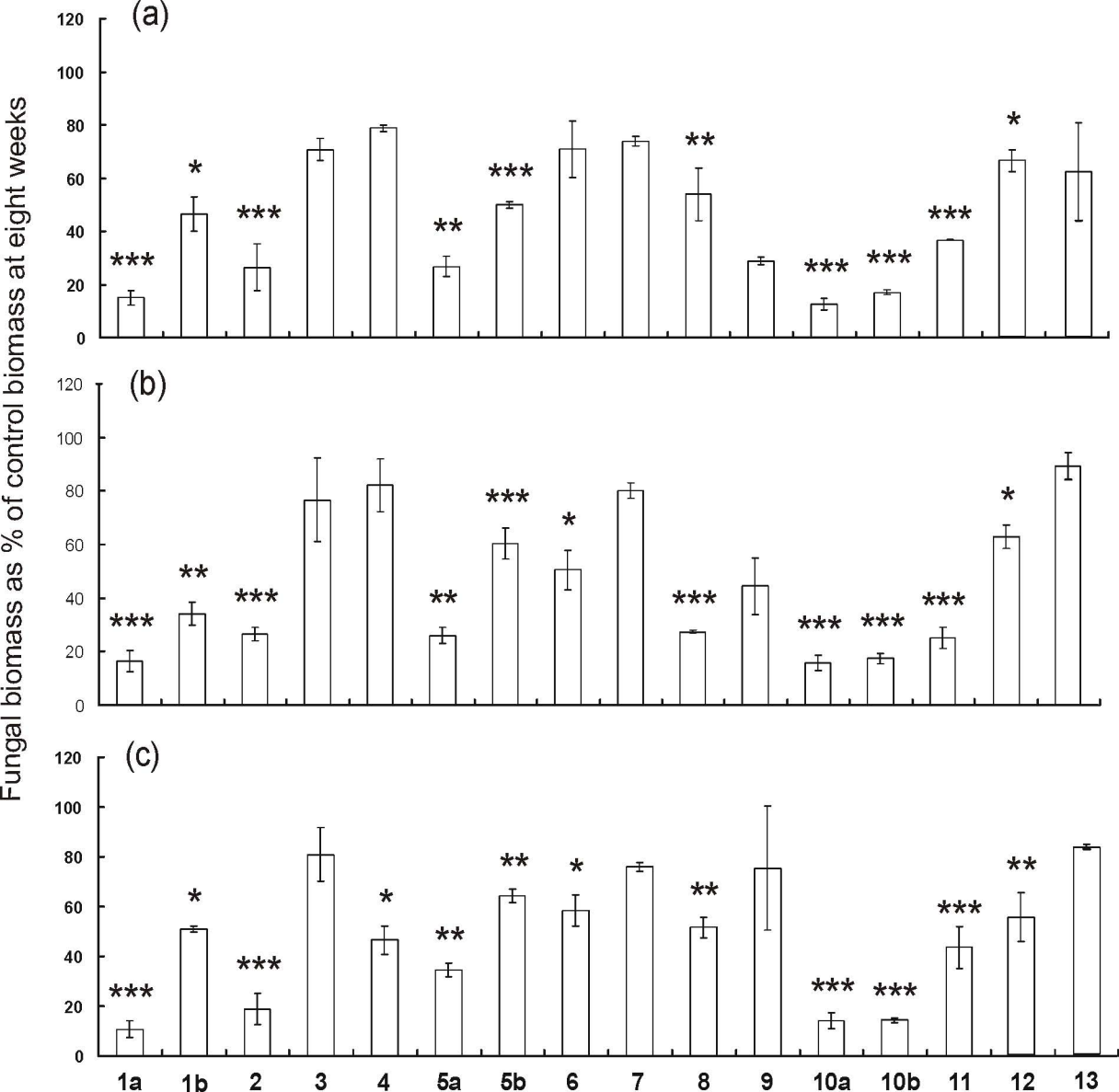
Isolate	Control biomass (mg)
Amanita muscaria 1	82.5 (2.9)
Amanita muscaria 2	16.3 (3.1)
Amanita rubescens	18.9 (1.8)
Amanita vaginata	32.9 (2.7)
Boletus badius	30.7 (5.4)
Cenococcum geophilum 1	54.0 (8.2)
Cenococcum geophilum 2	47.4 (2.7)
Hebeloma crustuliniforme	25.9 (2.4)
Laccaria amethystina	42.9 (4.8)
Laccaria laccata	44.2 (3.7)
Lactarius rufus	25.9 (17.5)
Paxillus involutus 1	53.4 (9.0)
Paxillus involutus 2	47.2 (5.9)
Suillus grevillei	44.4 (1.4)
Thelephora terrestris	24.0 (1.2)
Tricholoma aurantium	57.3 (5.4)

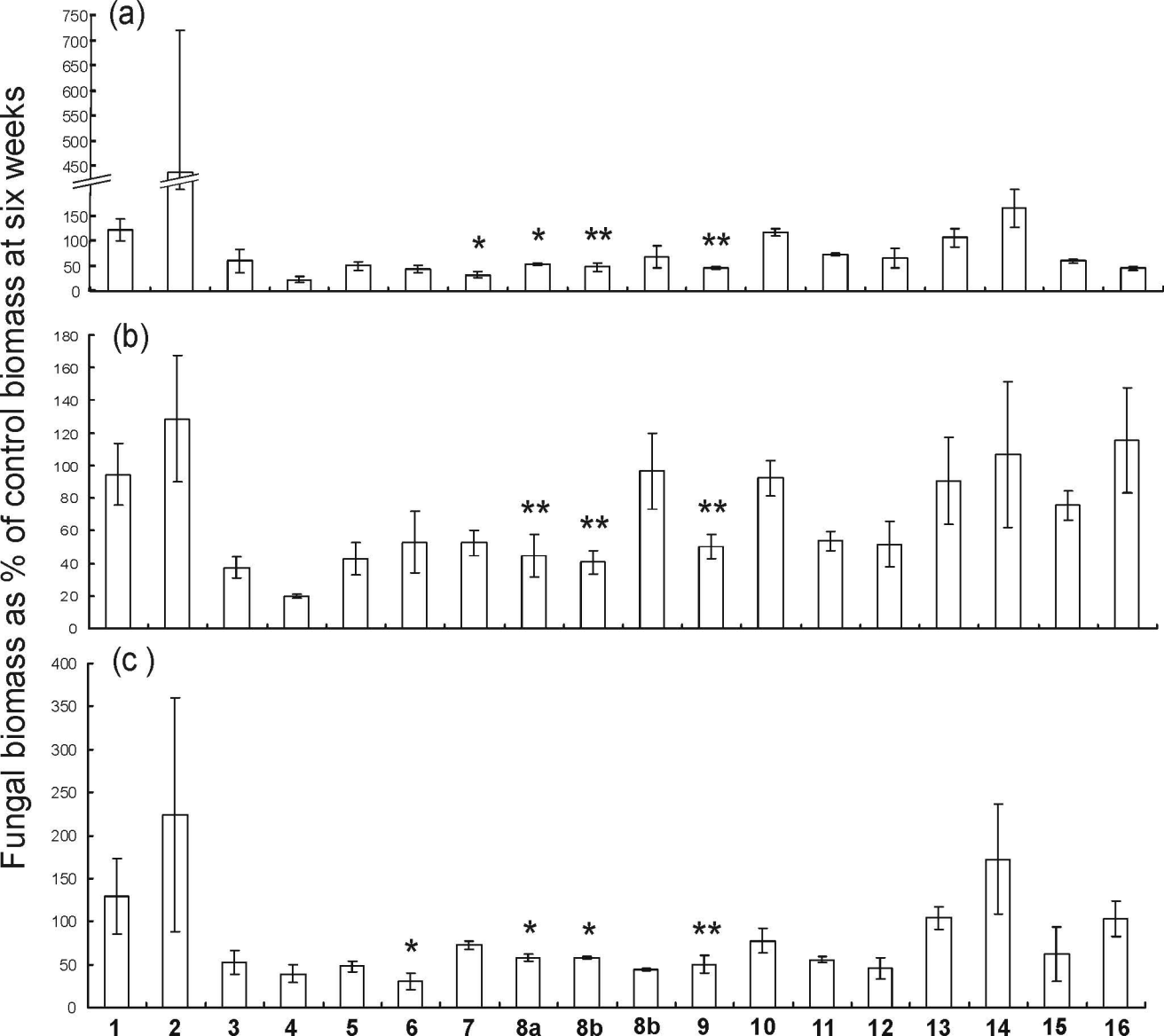
Table 3. Growth (mg dry wt) of saprotrophic isolates from control treatments after 6 weeks in liquid culture medium. Data represent the mean of three replicates (± SEM).

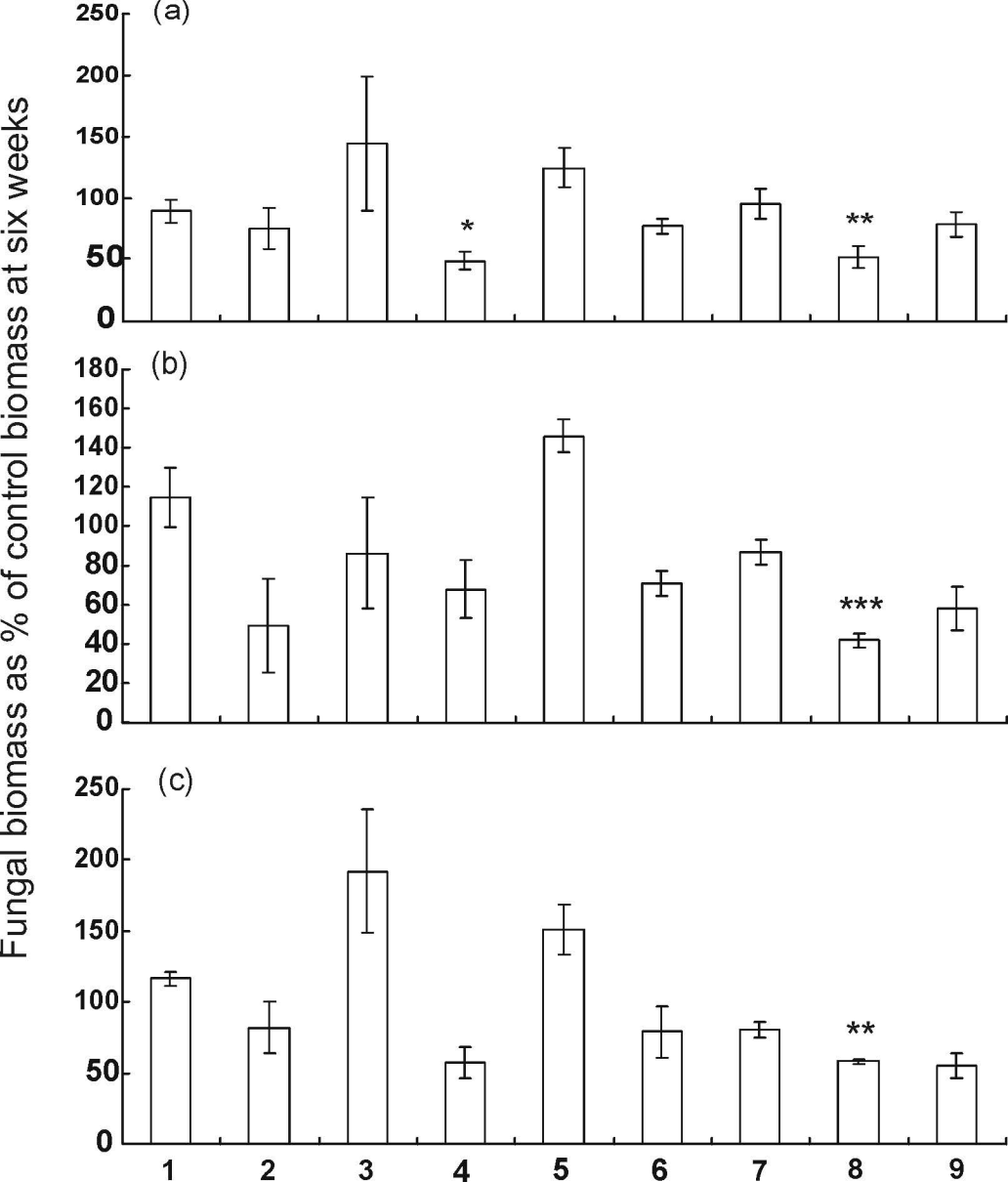
Isolate	Control biomass (mg)
Collybia butyracea	29.6 (17.0)
Collybia maculata	7.6 (2.5)
Collybia peronata	53.3 (5.1)
Cystoderma amianthinum	94.1 (39.8)
Marasmius androsaceus	73.8 (18.7)
Marasmius epiphyllus	96.4 (10.5)
Mycena cinerella	150.3 (39.4)
Mycena epipterygia	64.2 (5.7)
Mycena galopus 1	130.1 (9.1)
Mycena galopus 2	90.3 (7.7)
Mycena galopus 2	48.9 (10.4)
M. galopus var. *candida* 1	63.9 (4.3)
M. galopus var. *nigra* 1	47.0 (2.5)
Mycena purpureofusca	110.2 (34.2)
Mycena rubromarginata	27.0 (2.5)
Mycena sanguinolenta	96.8 (23.6)
Mycena vulgaris	50.9 (13.9)
Rickenella fibula	29.9 (3.8)

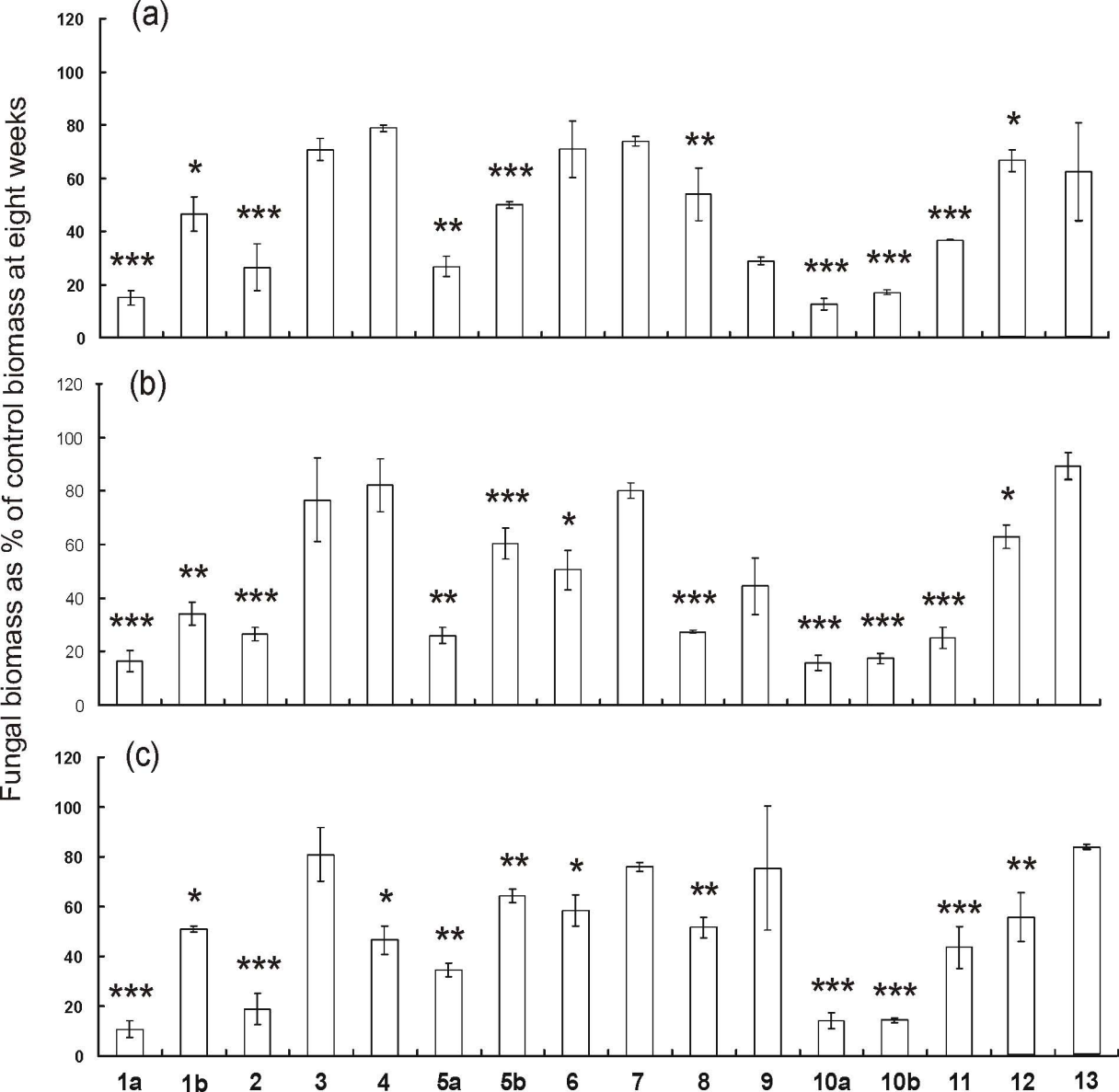
Table 4. Growth (mg dry wt) of saprotrophic *Mycena galopus* isolates from control treatments after 6 weeks in liquid culture medium. Data represent the mean of three replicates (± SEM).

Isolate	Control biomass (mg)
Mycena galopus 2	74.1 (2.6)
Mycena galopus 3	101.8 (8.9)
Mycena galopus 4	39.1 (10.4)
M. galopus var. *candida* 1	65.7 (5.7)
M. galopus var. *candida* 2	70.2 (6.2)
M. galopus var. *candida* 3	71.1 (1.6)
M. galopus var. *nigra* 1	67.3 (7.4)
M. galopus var. *nigra* 2	113.9 (7.8)
M. galopus var. *nigra* 3	87.3 (9.5)


(a)
(b)
(c)
Fungal biomass as % of control biomass at eight weeks
0
20
40
60
80
100
120
1a
1b
2
3
4
5a
5b
6
7
8
9
10a
10b
11
12
13


(a)
(b)
(c)
Fungal biomass as % of control biomass at six weeks
1
2
3
4
5
6
7
8a
8b
8b
9
10
11
12
13
14
15
16


Fungal biomass as % of control biomass at six weeks
(a)
250
200
150
100
50
0
*
**
(b)
180
160
140
120
100
80
60
40
20
0
***
(c)
250
200
150
100
50
0
**
1
2
3
4
5
6
7
8
9


(a)
(b)
(c)
Fungal biomass as % of control biomass at eight weeks
0
20
40
60
80
100
120
1a
1b
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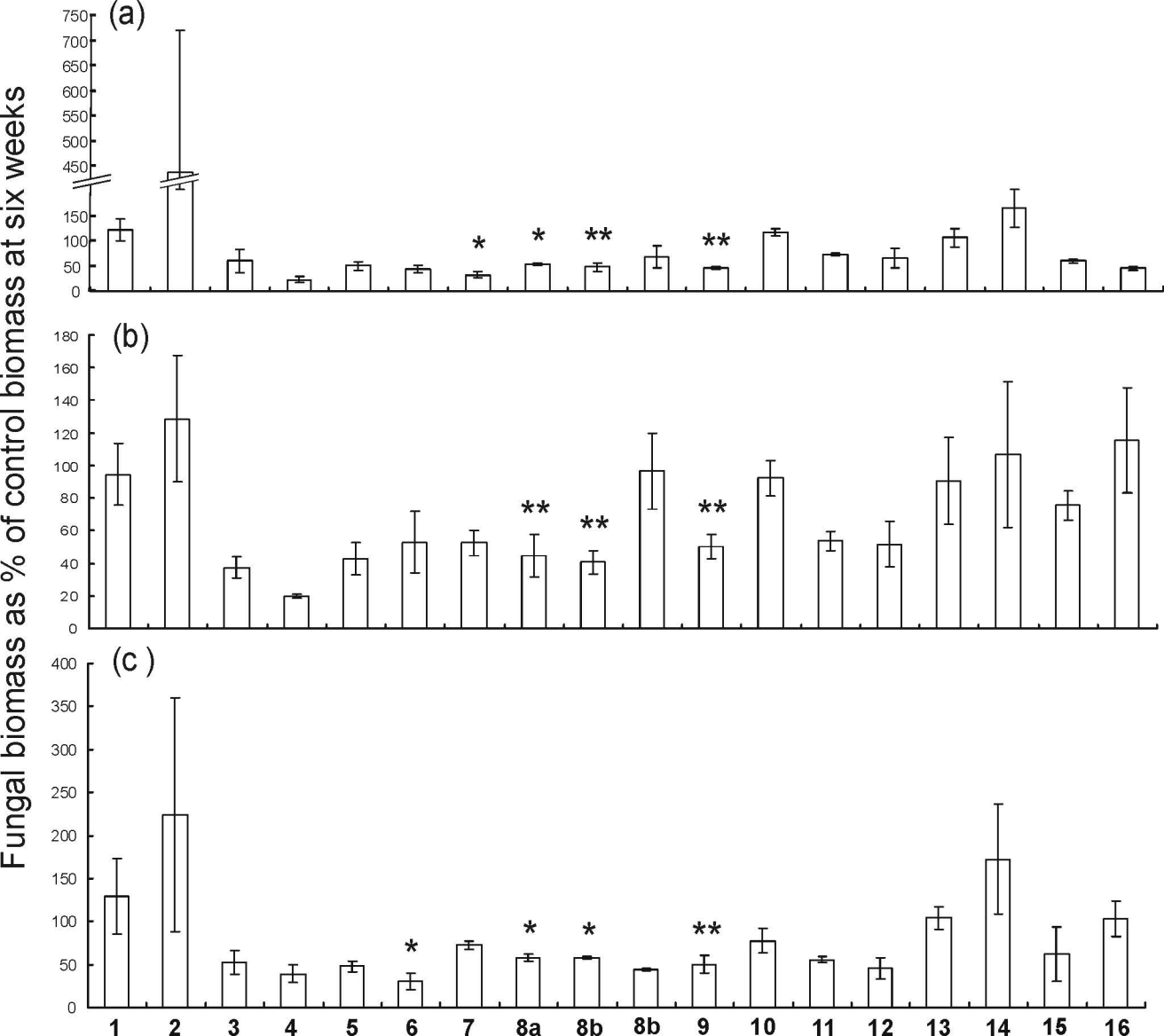
5a
5b
6
7
8
9
10a
10b
11
12
13


(a)
(b)
(c)
Fungal biomass as % of control biomass at six weeks
1
2
3
4
5
6
7
8a
8b
8b
9
10
11
12
13
14
15
16

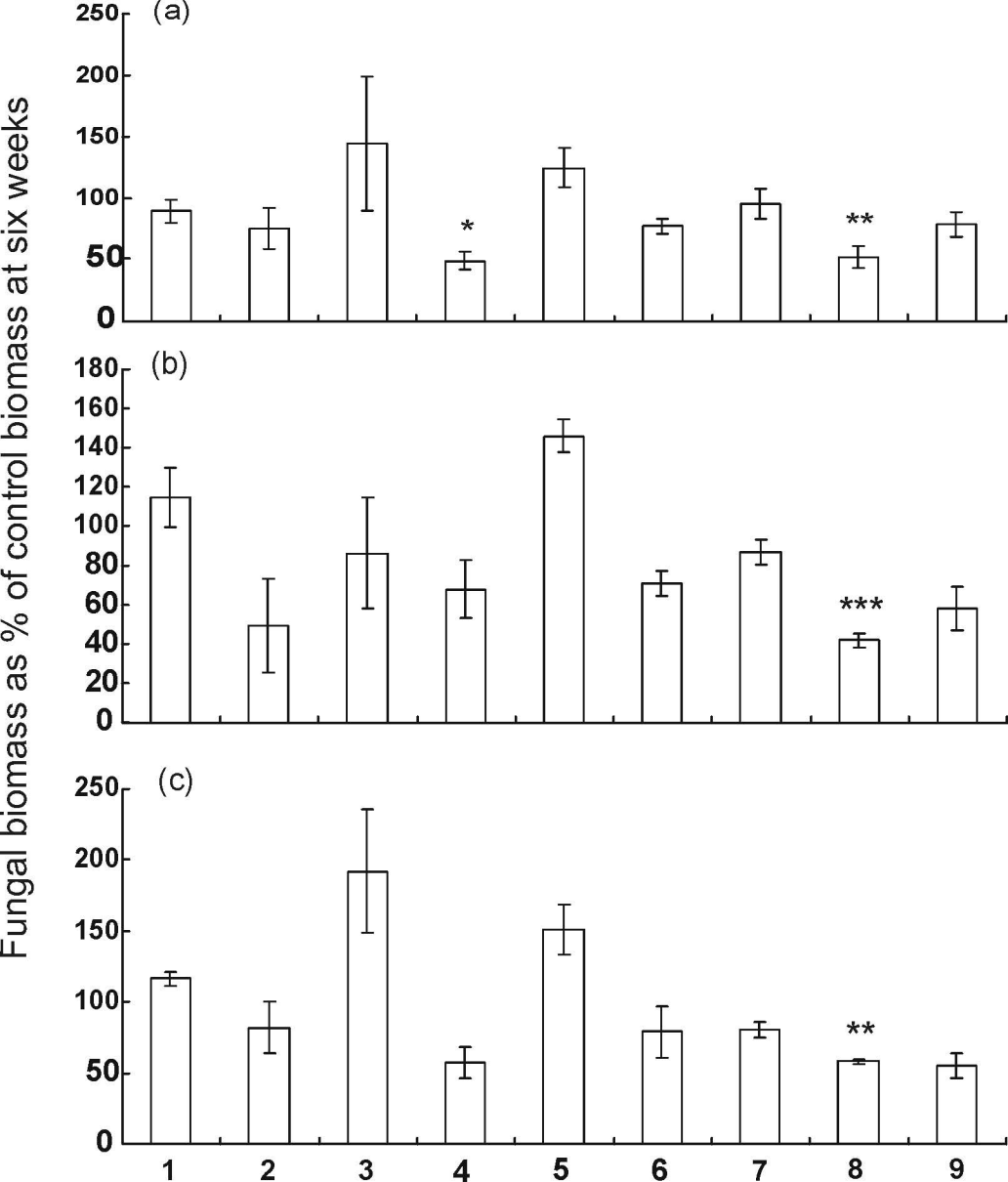

Fungal biomass as % of control biomass at six weeks
(a)
250
200
150
100
50
0
*
**
(b)
180
160
140
120
100
80
60
40
20
0
***
(c)
250
200
150
100
50
0
**
1
2
3
4
5
6
7
8
9

Table 1: List of fungal species and isolates tested. Culture code numbers refer to the number assigned to them in the collection of Dr Juliet C. Frankland unless otherwise stated. Where there is no culture code number, cultures were from freshly collected basidiome tissue.

Species	Culture code numbers	Substrate or tree species (where recorded)	Date isolated (where recorded)
Ectomycorrhizal isolates			
Amanita muscaria (L.) Lam.			
A. muscaria (Isolate 1)	Cardiff (A mus 0044)	Unknown	1985
A. muscaria (Isolate 2)	M9120	Unknown	1979
Amanita rubescens Pers.	Cardiff (Ar 006)	No details	1988
Amanita vaginata (Bull.) Lam.		*P. sylvestris*	2003
Boletus badius (Fr.) Fr.	M10627	Unknown	
Cenococcum geophilum Fr.			
C. geophilum (Isolate 1)	Cardiff (C geo 014)	Spruce litter	
C. geophilum (Isolate 2)	M10630	Unknown	1983
Hebeloma crustuliniforme (Bull.) Quél.	M10640	Unknown	1989
Laccaria amethystina Cooke	M8002	Unknown	1969
Laccaria laccata (Scop.) Cooke		Coniferous forest	2003
Lactarius rufus (Scop.) Fr.		*P. sitchensis*	2003
Paxillus involutus (Batsch) Fr.			
P. involutus (Isolate 1)		*P. sylvestris*	2003
P. involutus (Isolate 2)		Coniferous forest	2003
Suillus grevillei (Klotzsch) Singer	Cardiff (Se 010)	Unknown	
Thelephora terrestris Ehrh.	Cardiff (Thel t 12)	Unknown	1983
Tricholoma aurantium (Schaeff.) Ricken	M10661	*P. sitchensis*	1983
Saprotrophic isolates			
Collybia butyracea (Bull.) P. Kumm.	M9269 (b)	Mixed woodland litter	1981
Collybia maculata (Alb. & Schwein.) P. Kumm.	M10277	*P. sitchensis* litter	1989
Collybia peronata (Bolton) P. Kumm.	M10330	Unknown	1991
Cystoderma amianthinum (Scop.) Fayod	M9195	*P. sitchensis* litter	1977
Marasmius androsaceus (L.) Fr.	M10556	*P. sitchensis* litter	1998
Marasmius epiphyllus (Pers.) Fr.	M10263	Mixed woodland litter	1989
Mycena cinerella (P. Karst) P. Karst	M9235	Unknown	1980
Mycena epipterygia (Scop.) Gray	M9083	*P. abies* litter	1976
Mycena galopus (Pers.) P. Kumm			
M. galopus (Isolate 1)	M10500	*P. sitchensis* litter	1997
M. galopus (Isolate 2)	M10501	*P. sitchensis* litter	1997
M. galopus (Isolate 3)	M10502	*P. sitchensis* litter	1997
M. galopus (Isolate 4)	M10550	*P. sitchensis* litter	1998

M. galopus var. *candida* J. E. Lange			
M. galopus var. *candida* (Isolate 1)	M10505	*P. sitchensis* litter	1997
M. galopus var. *candida* (Isolate 2)	M9077	*P. abies* litter	1976
M. galopus var. *candida* (Isolate 3)	M10514	*P. sitchensis* litter	1997
M. galopus var. *nigra* Rea			
M. galopus var. *nigra* (Isolate 1)	M10504	*P. sitchensis* litter	1997
M. galopus var. *nigra* (Isolate 2)	M9092	*P. abies* litter	1976
M. galopus var. *nigra* (Isolate 3)	M10513	*P. sitchensis* litter	1997
Mycena purpureofusca (Peck) Sacc.	M9262b	*P. sylvestris* litter	1981
Mycena rubromarginata (Fr.) P. Kumm	M9286	*P. sylvestris* litter	1981
Mycena sanguinolenta (Alb. & Schwein) P. Kumm.	M10273	*P. sitchensis* litter	1989
Mycena vulgaris (Pers.) P. Kumm.	M10280	*P. sitchensis* litter	1989
Rickenella fibula (Bull.) Raithelh.	M10283	*P. sitchensis* litter	1989

Table 2. Growth (mg dry wt) of ectomycorrhizal isolates from control treatments after 8 weeks in liquid culture medium. Data represent the mean of three replicates (± SEM).

Isolate	Control biomass (mg)
Amanita muscaria 1	82.5 (2.9)
Amanita muscaria 2	16.3 (3.1)
Amanita rubescens	18.9 (1.8)
Amanita vaginata	32.9 (2.7)
Boletus badius	30.7 (5.4)
Cenococcum geophilum 1	54.0 (8.2)
Cenococcum geophilum 2	47.4 (2.7)
Hebeloma crustuliniforme	25.9 (2.4)
Laccaria amethystina	42.9 (4.8)
Laccaria laccata	44.2 (3.7)
Lactarius rufus	25.9 (17.5)
Paxillus involutus 1	53.4 (9.0)
Paxillus involutus 2	47.2 (5.9)
Suillus grevillei	44.4 (1.4)
Thelephora terrestris	24.0 (1.2)
Tricholoma aurantium	57.3 (5.4)

Table 3. Growth (mg dry wt) of saprotrophic isolates from control treatments after 6 weeks in liquid culture medium. Data represent the mean of three replicates (± SEM).

Isolate	Control biomass (mg)
Collybia butyracea	29.6 (17.0)
Collybia maculata	7.6 (2.5)
Collybia peronata	53.3 (5.1)
Cystoderma amianthinum	94.1 (39.8)
Marasmius androsaceus	73.8 (18.7)
Marasmius epiphyllus	96.4 (10.5)
Mycena cinerella	150.3 (39.4)
Mycena epipterygia	64.2 (5.7)
Mycena galopus 1	130.1 (9.1)
Mycena galopus 2	90.3 (7.7)
Mycena galopus 2	48.9 (10.4)
M. galopus var. *candida* 1	63.9 (4.3)
M. galopus var. *nigra* 1	47.0 (2.5)
Mycena purpureofusca	110.2 (34.2)
Mycena rubromarginata	27.0 (2.5)
Mycena sanguinolenta	96.8 (23.6)
Mycena vulgaris	50.9 (13.9)
Rickenella fibula	29.9 (3.8)

Table 4. Growth (mg dry wt) of saprotrophic *Mycena galopus* isolates from control treatments after 6 weeks in liquid culture medium. Data represent the mean of three replicates (± SEM).

Isolate	Control biomass (mg)
Mycena galopus 2	74.1 (2.6)
Mycena galopus 3	101.8 (8.9)
Mycena galopus 4	39.1 (10.4)
M. galopus var. *candida* 1	65.7 (5.7)
M. galopus var. *candida* 2	70.2 (6.2)
M. galopus var. *candida* 3	71.1 (1.6)
M. galopus var. *nigra* 1	67.3 (7.4)
M. galopus var. *nigra* 2	113.9 (7.8)
M. galopus var. *nigra* 3	87.3 (9.5)